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Chloroplast heteroplasmy is stabilized by an amber-suppressor tryptophan tRNA_{CUA}

(*Chlamydomonas reinhardtii*/organelle genetics/balanced polymorphism/polyploidy/ribulose-1,5-bisphosphate carboxylase/oxygenase)

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ABSTRACT Photosynthesis-deficient mutants of the green alga *Chlamydomonas reinhardtii* were previously shown to arise from nonsense mutations within the chloroplast *rbcL* gene, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39). Photosynthesis-competent revertants of these nonsense mutants have often been found to be stably heteroplasmic, giving rise to both mutant and revertant cells during mitotic or meiotic divisions under nonselective growth conditions. It was proposed that the stable heteroplasmy might arise from a balanced polymorphism of suppressor and wild-type alleles of a tRNA gene maintained within the polyploid chloroplast genome. In the present study, we have focused on determining the molecular basis for the heteroplasmy of one such revertant, named R13-3C, which was recovered from the 18-7G amber (UAG) mutant. Restriction-enzyme analysis and DNA sequencing showed that the amber mutation is still present in the *rbcL* gene of the revertant strain. In contrast, DNA sequencing of the suspected tRNA^{Trp} gene of the revertant revealed a mutation that would change its CCA anticodon to amber-specific CUA. This mutation was found to be heteroplasmic, being present in only 70% of the tRNA^{Trp} gene copies. Under nonselective conditions, the suppressor mutation was lost from cells that also lost the revertant phenotype. We conclude that stable heteroplasmy can arise as a balanced polymorphism of organellar alleles. This observation suggests that additional tRNA suppressors may be identified due to their heteroplasmic nature within polyploid genomes.

Polyploid genomes of organelles are generally viewed as consisting of identical copies of a single DNA sequence. This homoplasmy of organellar genes could be easily maintained by the predominantly uniparental inheritance of chloroplasts and mitochondria (1, 2). Nevertheless, heteroplasmic genomes have been detected as restriction-fragment length polymorphisms (3, 4) or as phenotypes that segregate during mitotic divisions (1). Whereas most heteroplasmic markers segregate rapidly to form homoplasmic cells (5), cases of stable heteroplasmy are known in various organisms ranging from algae and yeasts to higher plants and humans (6–9). In the unicellular green alga *Chlamydomonas reinhardtii*, photosynthesis-competent revertants of ribulose-1,5-bisphosphate (Rbu-*P*₂) carboxylase/oxygenase (EC 4.1.1.39) large-subunit mutants were recovered that are stably heteroplasmic for mutant and revertant phenotypes (6, 10). It was proposed that a balanced polymorphism of wild-type and mutant alleles of a chloroplast suppressor gene might be responsible for the stable heteroplasmy (6, 10).

Rbu-*P*₂ carboxylase/oxygenase catalyzes photosynthetic CO₂ fixation. It exists within the chloroplasts of plants and green algae as a hexadecameric holoenzyme composed of

equal numbers of chloroplast-encoded large subunits and nucleus-encoded small subunits (11). Amber (UAG) and opal (UGA) nonsense mutations have been defined within the chloroplast large-subunit gene (*rbcL*) of *C. reinhardtii* (12, 13), and, like all photosynthesis-deficient mutants (14), the nonsense-mutant strains have light-sensitive acetate-requiring phenotypes (12). In addition, they lack Rbu-*P*₂ carboxylase/oxygenase holoenzyme due to the rapid degradation of truncated or unassembled subunits (13). Photosynthesis-competent revertants of the nonsense mutants were found to be heteroplasmic, yielding both acetate-requiring (mutant) and wild-type (revertant) colonies after growth under nonselective conditions (acetate medium in the dark) (6, 10). Whereas no homoplasmic revertant segregant could be obtained after repeated cloning or crosses, all acetate-requiring segregants quickly became homoplasmic (6, 10). It was further shown that a revertant strain of the opal mutant retained the UGA mutation in all copies of its *rbcL* gene, but it synthesized both truncated and normal-length large subunits in pulse/chase experiments (15). Thus, it was proposed that heteroplasmy might arise from a chloroplast suppressor-tRNA gene maintained as a balanced polymorphism with its wild-type allele (10, 15). The suppressor allele would restore Rbu-*P*₂ carboxylase/oxygenase, but the wild-type allele would be required for normal protein synthesis. Under nonselective conditions (acetate medium in the dark), the suppressor-tRNA allele would be lost due to random segregation and genetic selection to give rise to homoplasmic acetate-requiring segregants.

In the present report, we have tested this hypothesis by investigating the molecular basis for heteroplasmic suppression of the 18-7G *rbcL* nonsense mutation (10, 12). This mutation changes the UGG codon for large-subunit Trp-66 to a UAG amber codon (13). Suppression of amber was likely to occur via a mutant tRNA^{Trp} because the 18-7G mutation occurred in a conserved region of *rbcL* (11) and the heteroplasmic revertants had catalytically-normal Rbu-*P*₂ carboxylase/oxygenase (10). Furthermore, since there is only one tryptophan codon and one tRNA^{Trp} gene (*trnW*) within the *C. reinhardtii* chloroplast genome (16), tRNA^{Trp}-mediated suppression would likely require a heteroplasmic mechanism.

MATERIALS AND METHODS

Strains and Culture Conditions. Wild-type *C. reinhardtii* 2137 *mt*⁺ (14) and *rbcL* mutant 18-7G *mt*⁺ (12, 13) are routinely grown with 10 mM acetate medium in darkness (14). Photosynthesis-competent revertant R13-3C was selected from mutant 18-7G (10). To maintain the revertant phenotype (6), this heteroplasmic revertant must be grown with minimal medium (without acetate) in the light (80 microeinsteins per m²

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Abbreviation: Rbu-*P*₂, ribulose 1,5-bisphosphate.

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per s). For experimental procedures, cells were grown in 50 or 500 ml of liquid medium on a rotary shaker at 25°C to a density of about 2×10^6 cells per ml.

Chloroplast DNA Isolation and Cloning. Total DNA was purified from 2 liters of cell culture (17) and then fractionated on NaI gradients to isolate chloroplast DNA (18). Following standard methods (19), *EcoRI*-digested chloroplast DNA was separated on a 0.8% agarose gel, and the ≈ 1900 base-pair (bp) *Eco29* fragment (18), containing *trnW* (16), was excised and purified by electroelution. This fragment was inserted into dephosphorylated plasmid pUC18 (20), which was then used to transform *Escherichia coli* XL1-Blue (21) by electroporation (22). The resulting plasmid was named p*Eco29*.

PCR Amplification and Cloning. Oligonucleotide primers were obtained from Research Genetics (Huntsville, AL) or the University of Nebraska Oligonucleotide Synthesis Facility. These included oligonucleotides 1030–1049 (GACT-GATAAGACAAGTACAT) and 1506–1485 (CGATTGGG-TAAGCTACGTAAGC) for amplifying an *rbcL* subfragment (23), and oligonucleotides 160–179 (CAATTTGTAGT-GAAATACTC) and 442–423 (GCACATAAGGACCTAG-GCTC) for amplifying *trnW* (16). PCR amplification was performed in a 100- μ l reaction mixture containing 50 pmol of each of the two primers, 2.5 units of *Taq* DNA polymerase (Cetus), 10 ng of chloroplast DNA, 200 μ M each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.3 (24). Amplification was performed for 25 cycles, each consisting of 1 min of denaturation at 95°C, 1 min of primer annealing at 37°C, and 3 min of primer extension at 72°C. The products were either digested or cloned directly. For cloning, the fragment ends were filled in with the Klenow fragment of DNA polymerase (19) and ligated with *HincII*-digested pBlue-script II (SK)– (Stratagene). Plasmids were transformed as noted above. The plasmid that resulted from cloning the full-length PCR product of *trnW* was named pW.

DNA Hybridization. Following standard methods (19), *Nla* III-digested PCR product from the *rbcL* gene was separated on a 1.5% agarose gel, blotted to nylon (Hybond-N; Amersham), and probed with a 5'-end ^{32}P -labeled synthetic oligonucleotide (bases 1506–1485). The filter was then subjected to autoradiography.

DNA Sequencing. Plasmid DNA was isolated by the boiling method (19), denatured in 0.2 M NaOH/2 mM EDTA at 37°C for 30 min, and neutralized with 0.1 vol of 3 M sodium acetate, pH 5.2. DNA sequencing (1 μ g of DNA per reaction) was performed by the dideoxynucleotide method (25) using Sequenase (United States Biochemical), deoxyadenosine 5'-[α - ^{35}S]thiotriphosphate, and forward/reverse primers or the synthetic oligonucleotides described above.

RESULTS

Nonsense-Mutation Homoplasmy. Photosynthesis-competent revertant R13-3C has maintained stable heteroplasmy for more than 5 years of vegetative growth on minimal medium in the light (10). To provide evidence that the revertant strain was homoplasmic for the original 18-7G *rbcL* mutation, we took advantage of the fact that this amber mutation (TGG \rightarrow TAG) eliminates an *Nla* III restriction-enzyme site (CATG \downarrow \rightarrow CATA) (13). A 477-bp fragment, bases 1030–1506 relative to the *rbcL*-containing *Eco14* map fragment (18, 23), was PCR amplified from wild-type, mutant 18-7G, and revertant R13-3C chloroplast DNA. *Nla* III would cut the wild-type product only after base 1387, generating fragments 358 and 119 bp long. As shown in Fig. 1, *Nla* III digestion of the wild-type PCR product was confirmed by detecting the 119-bp fragment with a ^{32}P -labeled oligonucleotide identical with *rbcL* bases 1506–1485. Furthermore, as expected, the PCR product from mutant 18-7G chloroplast DNA was not digested by *Nla* III (Fig. 1). The 477-bp

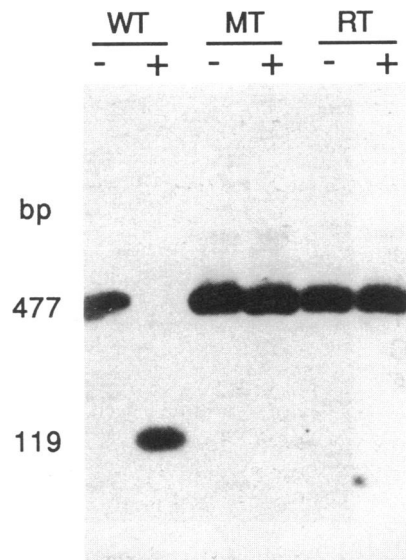


FIG. 1. Homoplasmy of the 18-7G *rbcL* mutation. A 477-bp fragment of *rbcL* was amplified from wild-type (WT), mutant 18-7G (MT), or revertant R13-3C (RT) chloroplast DNA. The products were then digested with *Nla* III; –, without digestion; +, with digestion. The samples were separated, blotted, and probed with a ^{32}P -labeled oligonucleotide homologous to the 3' end (119 bp) of the fragment. The 18-7G amber (UAG) mutation eliminates the *Nla* III site (13, 23).

fragment amplified from revertant R13-3C chloroplast DNA was also found to lack the *Nla* III site, suggesting that the original amber mutation is still present and homoplasmic (Fig. 1). PCR amplification and *Nla* III digestion of the wild-type and revertant R13-3C *rbcL* sequences were repeated three times, but no wild-type fragments or additional fragments were detected within revertant R13-3C, even with longer exposures of the autoradiograms. To further confirm the presence of the amber mutation within the *rbcL* gene of revertant R13-3C, four independent clones of the 477-bp PCR fragment were sequenced. These sequences were identical with the amber-mutant *rbcL* sequence of mutant 18-7G.

Suppressor-Mutation Heteroplasmy. Having provided evidence that revertant R13-3C was homoplasmic for the original *rbcL* nonsense mutation, we were encouraged to pursue our original hypothesis by investigating the *trnW* gene of the revertant strain. Since the proposed suppression mechanism predicted that revertant R13-3C would contain both mutant and wild-type *trnW* alleles (10, 15), it was necessary to sequence a number of independent *trnW* clones. Furthermore, we were concerned that the suppressor might also be lethal if expressed in *E. coli*. Therefore, a 283-bp fragment (bases 160–442) within *EcoRI* restriction-map fragment *Eco29* (16, 18) was amplified, but the PCR product was digested at a unique *Dde* I cut site that occurs after base 235. Since *trnW* is located between bases 230 and 302 (16), this procedure would effectively eliminate the potential expression of the gene. The resulting 5' promoter (bases 160–235) and 3' anticodon (bases 236–442) halves of the *trnW* fragment were cloned as a mixture, and independent clones were isolated at random and sequenced. Seventeen 5'-end clones were sequenced, but all of them had wild-type sequences. However, as shown in Fig. 2, when 28 3'-end clones were sequenced, 20 were found to contain a mutation that would change the tRNA^{Trp} CCA anticodon to amber suppressor CUA. We have named this suppressor mutation *trnW*-S13-3C. The remaining 8 clones had wild-type sequences, indicating that revertant R13-3C is, in fact, heteroplasmic for *trnW* alleles.

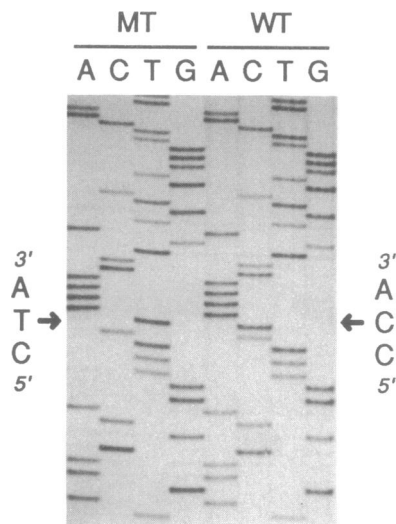


FIG. 2. Heteroplasmy of mutant (MT) and wild-type (WT) *trnW* alleles in revertant R13-3C. Twenty-eight independent copies of the anticodon region of the *trnW* gene were cloned and sequenced from revertant R13-3C. Twenty contained a C → T mutation at position 264 that would change the CCA anticodon of tRNA^{Trp} to amber suppressor CUA (16). The remaining copies contained the wild-type sequence. Sequencing ladders of representative *trnW* clones having either of these two sequences are compared in the figure.

To test whether the suppressor tRNA was lethal to *E. coli*, we also cloned intact *trnW* sequences from revertant R13-3C. However, the ratio of suppressor to wild-type tRNA alleles proved to be the same as for the cloned partial sequences when either the 283-bp PCR product or chloroplast *Eco29* fragment was cloned (Table 1). Although *C. reinhardtii trnW* has -35 and -10 promoter sequences similar to those in bacteria (16, 26), there may be additional requirements for its expression in *E. coli*. Alternatively, the suppressor tRNA may not compete with translation termination to an extent great enough to cause lethality. Nevertheless, since the same frequency of *trnW* suppressor alleles was observed with three different cloning methods (Table 1), it is likely that this is also the frequency of the S13-3C suppressor allele within the population of revertant-R13-3C cells.

Loss of the *trnW* Suppressor Mutation. To show that the *trnW* anticodon mutation was linked to the revertant phenotype, we investigated a homoplasmic acetate-requiring segregant of R13-3C. To obtain this segregant strain, revertant R13-3C was grown in acetate medium in the dark for approximately six population doublings (6). The cells were plated onto acetate medium plates in the dark to obtain single

Table 1. Frequencies of mutant-anticodon alleles among *trnW* genes cloned from revertant R13-3C chloroplast DNA

Cloned fragment*	Total <i>trnW</i> genes sequenced		Mutant allele frequency†
	Mutant (CTA)	Wild-type (CCA)	
3'-End (PCR)	20	8	0.71
Full-length (PCR)	9	4	0.69
<i>Eco29</i> (genomic)	8	4	0.67

*A 283-bp PCR product of chloroplast DNA, containing *trnW*, was cut within the *trnW* gene (3'-end) or cloned directly (full-length). *Eco29* is an ≈1900-bp *EcoRI* fragment of the chloroplast genome that contains *trnW* (16, 18). It was isolated from purified chloroplast DNA and cloned directly.

†Contingency χ^2 analysis indicated that the frequencies of *trnW* suppressor alleles determined from 3'-end, full-length, and *Eco29* fragments were not significantly different ($P > 0.70$ in all cases).

colonies, which were then replica plated to minimal medium in the light. Approximately 2% of the colonies were found to have acetate-requiring phenotypes, which is expected for heteroplasmic revertants previously recovered from the amber mutant strain (10). One acetate-requiring segregant colony was picked at random, grown in the dark, and cloned again. After this second cloning cycle, greater than 99% of the colonies had acetate-requiring phenotypes. One segregant colony was picked at random and maintained on acetate medium in the dark. Chloroplast DNA was isolated from the segregant strain, and the 283-bp fragment containing *trnW* was PCR amplified as described above. This fragment was digested with *Dde* I to inactivate *trnW*, and the mixture of fragments was cloned. Twenty independent 3'-end (anticodon) clones were sequenced. In addition, the 283-bp PCR product was cloned directly, and 23 independent clones were sequenced. None of these sequences contained the S13-3C anticodon mutation. Thus, the loss of the revertant phenotype correlates with the loss of the *trnW* suppressor mutation.

DISCUSSION

Molecular Basis for Stable Heteroplasmy. The photosynthesis-deficient *C. reinhardtii* mutant 18-7G contains an amber (UAG) mutation within the chloroplast *rbcL* gene of Rbu-*P*₂ carboxylase/oxygenase (12, 13). Photosynthesis-competent revertant R13-3C was selected from the 18-7G mutant strain, and it was found to regain about 25% of the normal level of holoenzyme (10). Like other revertants of *rbcL* nonsense mutants (6, 10), R13-3C is stably heteroplasmic; under nonselective growth conditions, it segregates out mutant and revertant phenotypes during mitotic and meiotic divisions (10).

In the present study, we have shown that the R13-3C revertant strain still contains the original amber mutation within its *rbcL* gene. Evidence for the homoplasmy of this mutation was obtained by DNA sequencing, as well as by screening for an *Nla* III restriction-enzyme site that was eliminated by the amber mutation (Fig. 1). In contrast, subsequent analysis of the chloroplast *trnW* gene of revertant R13-3C identified a mutation (which we have named *trnW*-S13-3C) that would change the CCA anticodon of tRNA^{Trp} to amber-specific CUA (Fig. 2). Only about 70% of the *trnW* gene copies contained the mutation (Table 1), indicating that the chloroplast genome of revertant R13-3C is, in fact, heteroplasmic for suppressor and wild-type *trnW* alleles. Furthermore, the *trnW* suppressor mutation was lost from cells that also lost the revertant phenotype during vegetative segregation under nonselective conditions. It is apparent that revertant R13-3C has regained photosynthetic competence due to informational suppression mediated by mutant tRNA^{Trp}, but the continued presence of wild-type tRNA^{Trp} is also required for cell survival.

These results indicate that stable heteroplasmy can arise as a balanced polymorphism of organellar alleles (6, 10). Whereas this polymorphism is maintained by selection for suppressor and wild-type alleles of *trnW*, one can anticipate that other types of balanced polymorphisms could be promoted by the polyploidy of organellar genomes. In fact, otherwise lethal mutations might be easily maintained in organelles, suggesting that organellar genomes have greater potential for genetic variation than is usually implied by uniparental inheritance and vegetative segregation (2, 5). This is illustrated by the present case, in which a tRNA^{Trp} amber suppressor has been identified: tRNA^{Trp} suppressors have been recovered only rarely by classical genetic approaches (27–29).

Genetics of Heteroplasmic Suppression. Since *E. coli* has only one gene for tRNA^{Trp} (30), it is easy to understand why few tRNA^{Trp} nonsense suppressors have been recovered

(31). If the identity of the tRNA was changed, there would be no tRNA to decode the single codon (UGG) for tryptophan. Thus, tRNA^{Trp} anticodon suppressors have been recovered and maintained as recessive lethals harbored on F factors, phage, or plasmids (31–33). Plasmids have also been used for constructing tRNA^{Trp} nonsense suppressors in *Saccharomyces cerevisiae* (34–36). Our results suggest that suppressor mutations within plasmid-borne tRNA genes could also arise via a “heteroplasmic” mechanism. Selection for a balance between wild-type and suppressor alleles within a population of plasmids may allow the recovery of other rare tRNA suppressors.

To our knowledge, there has been only one previous report of an organellar tRNA suppressor mutation (37). In that case, an apparently homoplasmic anticodon-stem mutation of mitochondrial tRNA^{Ser} was found to suppress a +1 frameshift mutation within the *S. cerevisiae* *oxl* gene (37, 38). Whereas it is difficult to study organellar mutations in multicellular organisms, it is intriguing that common tRNA suppressors have been lacking from the organelle genetics of *S. cerevisiae* and *C. reinhardtii*. Increased suppressor lethality might be expected in chloroplasts and mitochondria because most tRNA genes are present as single copies and there may be just enough tRNAs encoded for deciphering the genetic code (39, 40). Furthermore, the unusual codon recognition rules, and absence of certain tRNA genes in some cases, may cause mitochondrial tRNA suppression to be especially lethal (39, 41). In contrast, heteroplasmic tRNA suppressors should be easily promoted by the polyploidy of either organellar genome (6, 10, 15), but they have also been rare. Perhaps heteroplasmic “revertants” have frequently been lost under nonselective conditions (6) or excluded from analysis in favor of genetically stable strains.

Stably heteroplasmic mitochondrial tRNA mutations have been implicated in several human diseases (9, 42–44). This raises the possibility that a heteroplasmic tRNA mutation may be suppressing a still undetermined primary mutation, thereby explaining the stability of the heteroplasmicity. Although none of the mitochondrial tRNA mutations occur in the anticodon, suppression might be mediated by other types of tRNA mutations (28, 37), and the heteroplasmic suppression mechanism is expected to employ otherwise-lethal (and unusual) tRNA mutations (6, 10, 15). One should consider this possibility prior to initiating gene therapy with wild-type alleles of mutant tRNA genes (45). If heteroplasmic suppression is occurring in an affected individual, this therapeutic approach may not benefit the patient.

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